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Tangle-Bearing Neurons Survive Despite Disruption of Membrane Integrity in a Mouse Model of Tauopathy

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Abstract

Neurofibrillary tangles (NFTs) are associated with neuronal loss and correlate with cognitive impairment in Alzheimer disease, but how NFTs relate to neuronal death is not clear. We studied cell death in Tg4510 mice that reversibly express P301L mutant human tau and accumulate NFTs using in vivo multiphoton imaging of neurofibrillary pathology, propidium iodide (PI) incorporation into cells, caspase activation and DNA labeling. We first observed that in live mice a minority of neurons was labeled with the caspase probe or with PI fluorescence. These markers of cell stress were localized in the same cells and appeared to be specifically within NFT-bearing neurons. Contrary to expectations, the PI-stained neurons did not die over a day of observation; the presence of Hoechst-positive nuclei in them on the subsequent day indicated that the NFT-associated membrane disruption suggested by PI staining and caspase activation do not lead to acute death of neurons in this tauopathy model. This unique combination of in vivo multiphoton imaging with markers of cell death and pathologic alteration is a powerful tool for investigating neuronal damage associated with neurofibrillary pathology.

Keywords

In vivo imaging; Propidium iodide; Tangles; Tauopathy; Two-photon microscopy

INTRODUCTION

Neurofibrillary tangles (NFTs) composed of abnormally hyperphosphorylated tau protein assembled into paired helical filaments are, along with senile plaques and neuronal loss, a major pathological hallmark of Alzheimer disease (AD) (1). Intraneuronal NFTs are also pathological hallmarks of tauopathies, a large group of disorders of cognition and movement that includes progressive supranuclear palsy, corticobasal degeneration, Pick disease and frontotemporal dementia and Parkinsonism linked to chromosome 17 (2). Although it is widely known that accumulation of insoluble tau aggregates correlates with disease progression, current understanding of the mechanisms involved is insufficient to conclude that there is a causal relationship between NFTs and neuronal death.

rTg4510 mice reversibly express P301L mutant human tau and develop neurofibrillary pathology, neuronal loss and memory impairment (3–5). In this model, NFTs appear as early as 2.5 months of age; neuronal loss is dramatic and approaches approximately 50% loss of cortical neurons by 8.5 months of age (5). Both tangle formation and neuron loss are region-specific and appear to be dissociated processes. For example, loss of neurons occurs before neurofibrillary lesions in the dentate gyrus; conversely, neurofibrillary pathology appears without major cell loss in other areas such as the basal ganglia (5). In the cortex and hippocampal CA fields, however, the extent of tangle accumulation and neuronal loss are correlated, suggesting either that tangles precede and lead to neuronal death or that they are independent but concurrent pathophysiological events.

We previously showed that it is possible to visualize NFT in the brains of live rTg4520 mice using 2-photon in vivo imaging through a cranial window. We developed a method to image caspase activation in these mice and our observations suggested that apoptosis-like mechanisms are responsible for their neuronal death (6). Caspase activation could be detected in vivo in a small percentage of neurons; there was nearly universal colocalization of caspase-positive neurons and NFTs but these neurons did not proceed to an acute apoptotic death over the subsequent hours of observation. We hypothesized that the caspase-positive neurons are under cellular stress but that they do not die immediately. Therefore, we postulated that using a later marker for cell death would be a better method for revealing all dying neurons.

In the present study, we sought to develop another marker that might reflect additional (i.e. non-apoptotic) cell death mechanisms and that would not be dependent upon enzymatic activation of a probe. We employed propidium iodide (PI), a widely used fluorescent marker of cell death in tissue culture assays (7). PI is excluded from cells with intact membranes but can access dead or dying cells. When PI is injected intravenously into mice subjected to middle cerebral artery occlusion, PI-positive neurons can be observed postmortem in association with ischemic damage (8). Thus, PI labels compromised neurons in mouse brains. Moreover, its fluorescence increases 20- to 30-fold upon binding to DNA so that background signal is low and positive cells are identified as having bright red nuclei (7). We also used the Hoechst fluorescent DNA-binding dye that diffuses freely through membranes and labels nuclei of living cells blue. We found that during in vivo imaging of 7- to 9-month-old pg4510 mice PI labels a small number of neurons. These neurons nearly universally also stain with the active caspase reagent and contain NFTs. In accord with our observations using the caspase reagent alone, neurons that are caspase- and PI-positive remained intact 24 hours after initial imaging, thereby supporting the concept that NFT-containing neurons are under substantial stress but do not acutely die.

MATERIALS AND METHODS

Surgery and Imaging

All animal experiments were carried out following National Institutes of Health and Massachusetts General Hospital Committee on Research Animal Care guidelines. We used 7- to 9-month-old rTg4510 mice that reversibly express P301L mutant human tau ($n = 6$) (3). The mice are bred by R. Pitstick and G. Carlson at McLaughlin Research Institute, Great Falls, Montana. Age-matched littermates that express only the activator transgene were used as negative controls ($n = 4$). A craniotomy was performed as described previously (9). Briefly, animals were anesthetized with isoflurane (0.5–2%, Baxter, Deerfield, IL) in balanced oxygen and a 6-mm circle was drilled on the dorsal surface of the skull (centered on the midline with the anterior tip of the circle immediately anterior to Bregma), and the 6 mm circle of encompassed skull removed. Before a glass coverslip was sealed over the craniotomy, dura were resected from the cortical surface and phosphate buffered saline (PBS) containing Thioflavin S (0.025% Thioflavin S, Sigma, St. Louis, MO), a fluorescent indicator of caspase

activation (FLICA indicators, 5× concentration, polycaspase indicator, Molecular Probes, Eugene OR) and PI (Molecular Probes, 10 µg/ml) was applied for 20 minutes. For longitudinal imaging, Hoechst 33342 (Invitrogen, Carlsbad, CA) solution in 1 mM PBS was applied with the other fluorescent markers (n = 2) and imaged the next day.

In vivo imaging was performed using a Bio-Rad 1024ES multiphoton microscope (Bio-Rad Laboratories, Hercules, CA) with 800 nm excitation (Ti:Sapphire laser: Mai Tai, Spectra-Physics, Mountain View, CA) for Thioflavin S, as described previously (10). We empirically found that 800 nm excites PI, caspase indicator and Thioflavin S optimally on our Mai Tai laser system. We used 750 nm excitation on the following day to detect Hoechst 33342 fluorescence. For quantification of NFTs and cell death markers, images were taken in a systematic random fashion through the window area to ensure unbiased sampling (11). For each animal, z-stacks from 8 different areas of 307.92- × 307.92- × 52 µm (representing 5% of the window area) were collected. Observations of the same neurons on consecutive days were possible using XY coordinates recorded for each high resolution (5×) image of cells of interest; lower resolution (1×) images of the surface of the brain were used as reference to identify the same site based on the blood vessels; 2× images of the same focal plane provided an overview of the area with neighboring cells.

After completion of the experiments, the animals were killed with an overdose of avertin (2,2,2-tribromoethanol in t-amyl alcohol [Sigma], 400 mg/kg). The brains were harvested, fixed in 4% paraformaldehyde containing 15% glycerol cryoprotectant for 2 to 3 days and then either sectioned or stored at 4°C in a 20% sucrose solution.

Microscope Parameters

1× and 2× images were taken as a location reference with a “normal” rate of scan speed. The rate of scan speed was “low” for high-resolution (5× and 10×) images to assess detailed cell morphology. Laser intensity was 30%, which was presumably low enough not to cause cell damage. PMT voltage was unchanged for all imaging sessions at 750 volts for the blue channel, 850 volts for the green channel and 1050 volts for the red channel. Imaging times were between 1 and 20 seconds depending on the depth of scan.

Image Post-Processing

Images were taken as z-stacks. All images were processed with ImageJ and presented as z-projections. Intensity, contrast and color balances were used to pseudo-color the images and the enhance quality of some images.

Postmortem Analysis

The brains were frozen and serial coronal sections 40 µm thick were cut on a freezing microtome. An unbiased stereologic counting method was used to determine the percentage of Thioflavin S-positive neurons containing a Hoechst-positive nucleus. The optical fractionator method was used as previously described (12). The image analysis system CAST (Olympus), mounted on an upright BX51 Olympus microscope with an integrated motorized stage (Prior Scientific, Rockland, MA) was used for outlining regions, sampling and counting. Tangle-bearing neurons with and without a visible nucleus were counted in a 21.8 × 21.8 × 36 µm counting frame with a guard volume of 2 µm at the edges of the section to avoid sampling tangles that had lost their nuclei during sectioning. Dissectors were placed using a meander sampling paradigm (a grid placed at random angles on the region of interest) to sample 2% of the tangles in the area under the cranial window.

Statistics

To assess whether the cells with PI-positive tangles observed *in vivo* died leaving “ghost tangles” (those without a health-appearing Hoechst-positive nucleus postmortem), we designated cells with PI-positive-tangles and ghost tangles as “dying” neurons. The Chi squared statistic from contingency table analysis (using the Stat View program, SAS Institute, Cary NC) was used to determine whether the percentage of putative dying tangle-bearing neurons (PI-positive) *in vivo* were the same percentage as those that are actually dying (as assessed by the absence of a nucleus associated with the tangle postmortem). Chi squared p value of <0.05 was considered significantly different, implying that PI-positive cells *in vivo* are not dying.

RESULTS

Small Numbers of Neurons with NFTs Contain Cell Death Markers

A small proportion of cells with NFTs (6%) had PI-positive nuclei indicating disruption of membrane integrity (Table, Fig. 1). To ensure that there was no interference between the compounds, we performed a control experiment by applying PI first, imaging PI-labeled cells, and then applying Thioflavin S or Hoechst and re-imaging the same brain area. Results with these sequential applications were consistent with our earlier findings (6) (data not shown). We also confirmed by stereological counts on postmortem tissue that the numbers of cells with PI uptake in the window area were comparable to the numbers found by multiphoton imaging (data not shown); this suggested that the relative rarity of PI-positive neurons detected was not due to insensitivity of the *in vivo* imaging methodology.

Simultaneous imaging of NFTs and caspase activity showed that a similarly small number of cells with tangles had some caspase activation. No labeled cells were detected by either method in non-transgenic littermate animals. The majority of NFT-containing neurons were not positive for cell death markers, indicating that the presence of NFTs does not necessarily lead the neuron to a cell death pathway.

The Cell Death Markers Colocalize and Appear to be Specifically Within Tangle-Bearing Neurons

In the majority of labeled cells, caspase indicator or PI was found in tangle-bearing neurons (Fig. 2A), indicating that NFTs are not only associated with caspase activation but also with loss of membrane integrity, a later hallmark of cell death. Using a green poly-caspase indicator in combination with PI (Fig. 2B), we determined whether the 2 cell death markers were colocalized. They were almost always seen together in cells (Fig. 2C), suggesting that activated caspase is associated with membrane destabilization. The caspase indicator was mostly within the cytoplasm whereas the PI label was exclusively the nucleus, thus allowing visualization of both markers in green NFT-bearing cells (Fig. 2D). Taken together, these data are consistent with our previous study showing caspase activation and they further show that caspase activation is associated with loss of membrane integrity in a minority of NFT-bearing neurons.

Death Marker-Positive Tangles Persist Over 24 Hours

Despite the presence of caspase activation, NFT-bearing neurons were previously found to be unchanged over hours of imaging (6). Here, we observed a similar absence of degeneration over hours when the cells were PI-positive.

To investigate whether cell death markers are associated with subsequent degeneration of NFT-bearing neurons, we re-imaged the same neurons 24 hours after initial imaging (Fig. 3). All tangle-bearing neurons, including those labeled with cell death markers on the first day of imaging, were detected at both time points. Prolonged excitation of fluorescent probes can lead

to relatively fast quenching, especially *in vivo* where anti-quenching agents cannot be applied. Thioflavin S fluorescence remained detectable despite light photobleaching, but PI fluorescence was rarely seen on the second day; caspase indicator fluorescence faded after several hours.

Since both cell death markers faded, we could not be certain whether the tangles that persisted on the second day were in living neurons that continued to have a nucleus, or whether the cells had died leaving behind “ghost tangles.” “Ghost tangles” in AD are observed in entorhinal cortex and hippocampus but rarely in neocortex. To address this, we used Hoechst dye as a marker of living cells. A solution of Hoechst 33342 was applied on the surface of the brain at the first imaging session. Since it is a highly lipophilic dye, Hoechst 33342 penetrated the brain tissue and crossed intact cell membranes. The next day, Hoechst fluorescence had reached layers II–III of the cortex where Thioflavin S-labeled NFTs were observed; it was detectable at a 750-nm excitation. No evidence of “ghost tangle” formation was observed, i.e. cells with NFTs associated with death markers on the first day of imaging contained Hoechst-positive nuclei on the second day (Fig. 3H). Postmortem analysis of the brains showed that only 1 out of 233 NFT-containing neurons did not contain a clearly stained Hoechst-positive nucleus (Table), confirming that “ghost tangles” are at most a rare occurrence in this model.

DISCUSSION

To investigate the relationships between the presence of NFTs and neuronal death, we imaged markers of these events in the brains in live mice in a model of tauopathy. Our observations suggest that only a very small proportion of neurons show signs of degeneration at any individual point in time, as might be expected for a neurodegenerative process that progresses over many months.

We hypothesized that caspase activation would be an early sign of cell distress due to apoptosis-like events and that PI staining of the nucleus (which is a widely used marker of dead and dying cells reflecting loss of membrane integrity) would be a later marker of neuronal death not dependent on a particular pathophysiological process. Our earlier studies suggested that a minority of neurons was caspase-positive and that these were associated with NFTs since they did not lead to immediate death over a short time period (6). Our calculations suggested that the number of caspase-positive cells was insufficient (by an order of magnitude) to account for the number of neurons dying each day. Therefore, sought to develop another marker of cell death that might provide insight into whether or not the caspase-positive NFT-containing neurons were really dying, and possibly reveal neurons undergoing non-caspase mediated death processes.

Our results show that PI can be used as an *in vivo* marker of loss of membrane integrity since there was a robust staining of neurons by multiphoton microscopy. These PI-positive neurons were almost certainly distressed since they nearly always contained NFTs and displayed both early and late markers of apoptosis; caspase activation and cell membrane disruption occurred together. Previously in the same model, we found that initiator and executioner caspases appeared to be activated in tangle-bearing neurons (6). Here, we found significant colocalization of caspase activation with cell permeabilization to PI associated caspase activation with downstream cell damage.

Our data show several surprising features. Although PI is a robust marker of cell distress *in vivo*, we observed only a very small number of neurons that were PI-positive at any imaging session. Based on cross-sectional stereological analysis collected from the entire neocortex, we calculate that there would be an approximate loss of 680 neurons per day in the volume we imaged, whereas we observed only 20% of that number to be PI-positive. In this estimate, we

assumed that all cortical regions undergo similar neuronal loss, since the entire cortical mantle shrinks considerably in these mice. If PI staining were transient, for example if neurons that were PI-positive died and were cleared rapidly by apoptosis-related mechanisms, this dissociation could be explained using a kinetic argument. However, our longitudinal imaging data show that PI-positive neurons persist for at least 24 hours, as judged by the continued presence of the neurofibrillary tangle, by our ability to detect Hoechst-positive nuclei 24 hours after observing PI staining, and the absence of ghost tangles. This surprising result suggests 2 major conclusions: first, that neurons containing tangles, caspase activation and even PI staining nonetheless can survive for longer than we would have expected based on *in vitro* use of these cell death markers. Wilde et al investigated the use of PI as a marker for acute degeneration *in vivo* in rat brain and found that PI-labeled CA3 pyramidal cells had no obvious histologically detectable cell damage for hours after stress was induced (13). After ischemic injury in mouse brain, intracerebroventricular administration of PI showed that most PI-labeled cells were not TUNEL-positive in areas with mild injuries; PI incorporation has been observed in cells that do undergo apoptosis in areas displaying more severe ischemic changes (8). Non-lethal caspase activation has been observed, however, in studies of synaptic plasticity and neuroprotection (14). A more recent study showed the limits of PI as a cell viability indicator for bacteria and that, in addition to nonviable cells, it also stains cells with cell cycle activity, a stage where cell membrane integrity is temporarily affected (15).

Our results reinforce the idea that tangle-containing neurons are much more likely than non tangle-bearing neurons to show signs of acute distress, including caspase activation and PI staining. The large majority of tangle-bearing neurons are not caspase- or PI-positive, however, suggesting that tangles are associated with but do not necessarily cause activation of cell death pathways. The mechanisms that allow the damaged tangle-bearing neurons to survive remain to be understood but suggest the possibility that endogenous transitory neuroprotective mechanisms are activated in tangle-bearing neurons.

One of our goals for developing PI as a marker of cell death was to better understand the relationship between tangle formation and cell death in this model. Our calculations, based on rate of neurodegeneration from cross-sectional studies of the rTg4520 mice, suggest that there is neuronal loss occurring in the cortex that is not accounted for by rare neurons that contain a tangle, and contains caspase and PI neurodegeneration markers. We are now trying to develop markers of earlier lesions, perhaps prior to tangle formation, to further explore this paradox.

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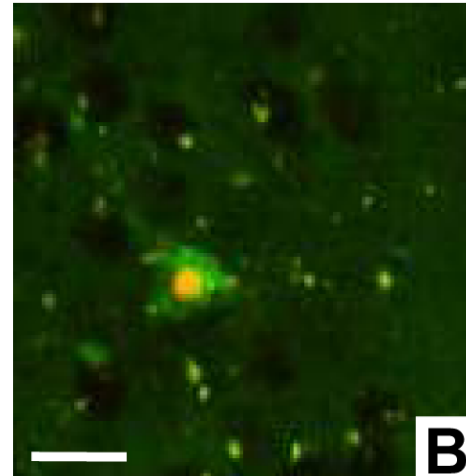
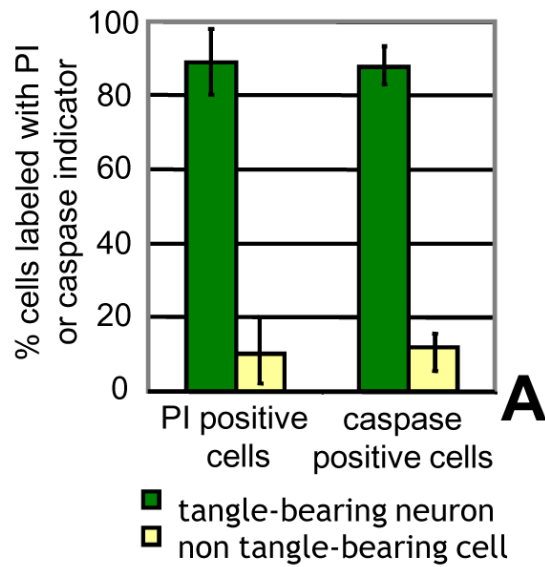
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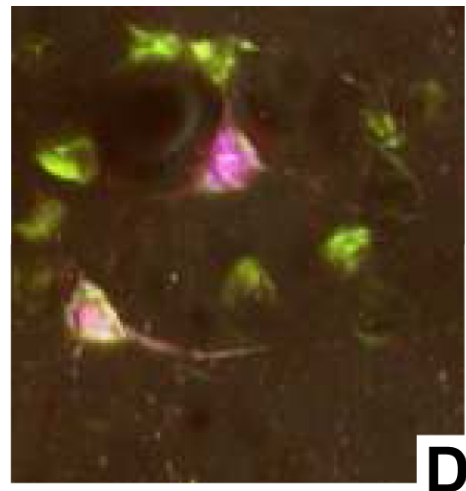
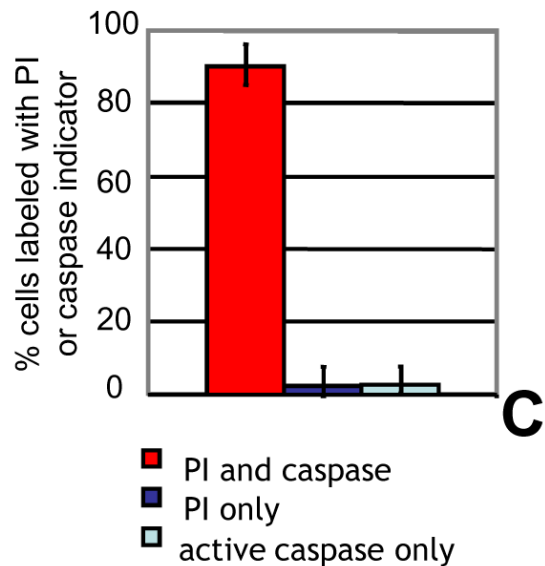
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Co-localization of markers and NFTs



■ casp. indicator ■ PI

PI and activated caspase co-localize



■ tangles ■ PI (nuclei)
casp. (cytopl.)

Figure 1.

In vivo imaging of neurofibrillary tangles (NFTs) and propidium iodide (PI) incorporation into cells. (A) PI incorporation in the brain of a live transgenic mouse. PI incorporation is a rare event and labeled nuclei (red) are detected almost exclusively in cells containing Thioflavin S-positive NFTs (green fluorescence). No positive nuclei were observed in non-transgenic animals. (B) PI exclusively labels the nucleus in NFT-bearing cells. Incorporation of this dye indicates disruption of membrane integrity. Scale bars: A = 20 μ m; B = 10 μ m.

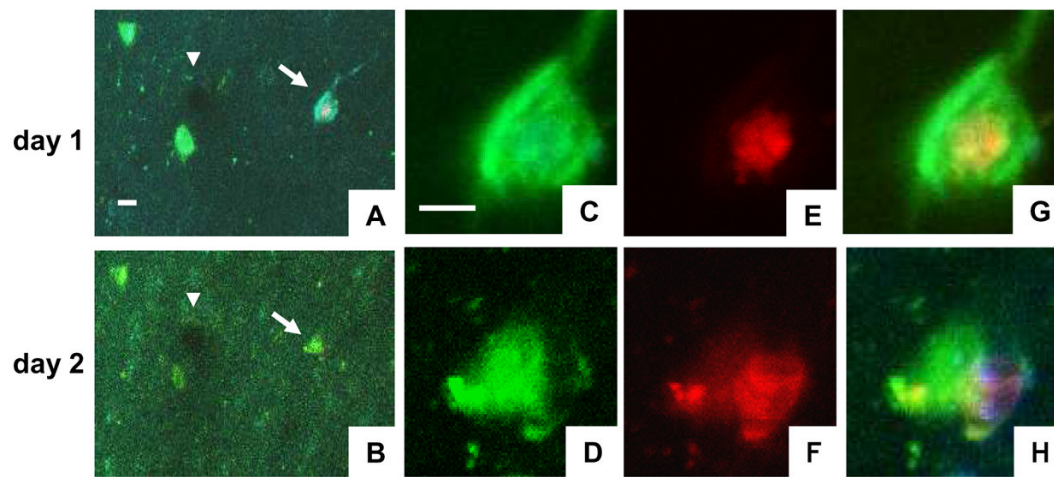


Figure 2.

Colocalization of cell death markers and neurofibrillary tangles. **(A)** The same numbers of cells with propidium iodide (PI) and activated caspase indicator labels are seen in tangle-bearing neurons. **(B)** Using a green caspase indicator (mostly cytoplasmic) with PI (exclusively nuclear) reveals colocalization of these cell death markers in a representative cell. **(C)** Double-labeled PI- and caspase indicator-positive cells outnumber single-labeled cells. **(D)** Both compartment-specific indicators are visualized in cells containing green Thioflavin S aggregates. Three colors are involved in this image. Scale bar = 10 μ m.

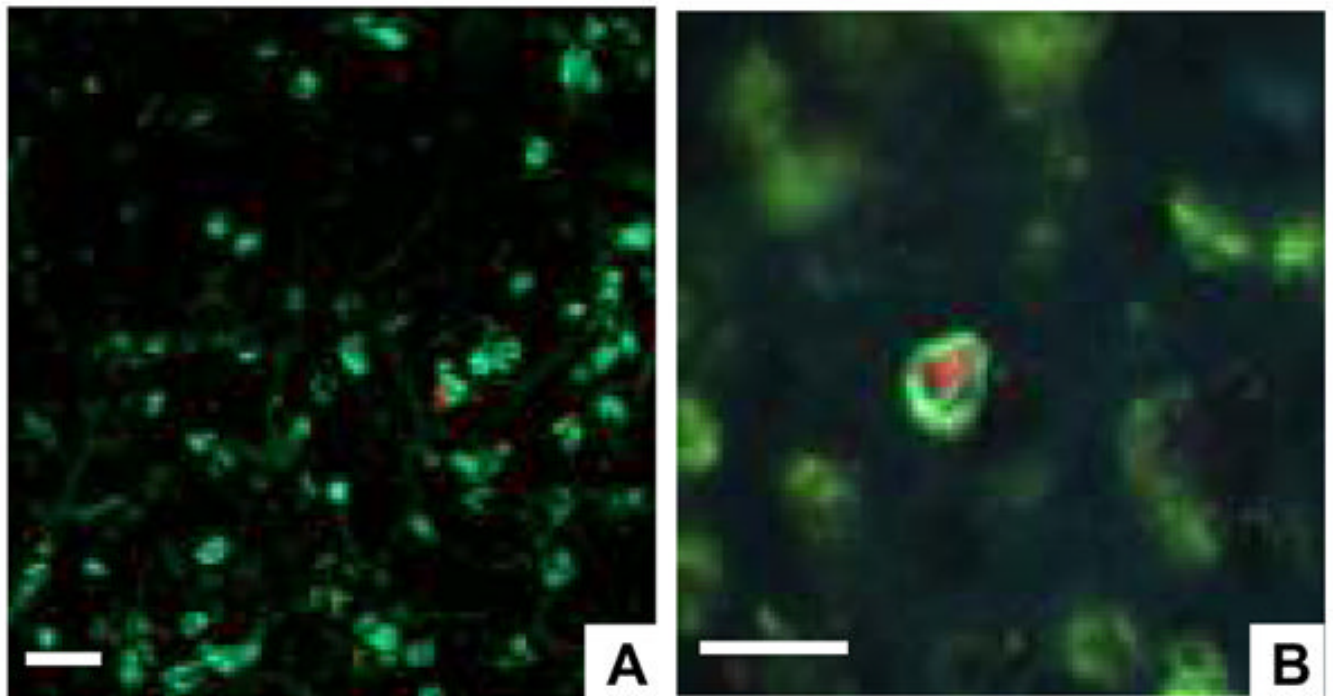


Figure 3.

Absence of degeneration in tangle-bearing neurons despite the presence of a cell death marker. Longitudinal *in vivo* imaging of neurons reveals that a tangle-bearing neuron labeled with propidium iodide (PI) (arrow, **A**) can be re-imaged on the following day (arrow, **B**). Arrowheads in **A** and **B** label the same blood vessel used as a landmark for finding this neuron. The neuron has Thioflavin S aggregates (pseudo-colored in green, **C**) and a PI-positive nucleus (**E**). It shows persistence of Thioflavin S aggregates over 24 hours (labeled in green at 800 nm, **D**), a healthy-looking Hoechst-positive nucleus (labeled in blue at 750 nm, **D**), and some remaining but faded PI labeling (**F**). Thioflavin S and PI labeling are merged (without detectable Hoechst labeling at this time) in (**G**) on the first day of imaging. Thioflavin S, Hoechst and PI are merged on the second day of imaging in (**H**). The blue Hoechst and red PI label result in the purple color in (**H**). Scale bars: **A**, **B** = 10 μ m; **C**–**H** = 5 μ m.

Table

Comparison Between the Presence of Cell Death Markers and “Ghost” Tangles

	<i>In vivo</i>	Postmortem
Number of tangle-bearing neurons counted	2265	233
Dying or dead tangle-bearing neurons	136 [*] (6%)	1 ^{**} (0.42%)

^{*}, Cells with a propidium iodide (PI)-positive nucleus; presumably a dying or dead cell

^{**}, Number of cells without a visible Hoechst-positive nucleus; a dead cell or “ghost tangle”.

This comparison indicates that tangle-bearing neurons are more likely to have a PI-stained nucleus in vivo than to be “ghost” tangles in post-mortem tissue. Chi Square value: $p < 0.0001$ in contingency table analysis.